Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Patrícia Alexandra Oliveira Pereira Kowalski, investigadora em Pós-doutoramento da Universidade de Aveiro, e do Doutor Mário Guilherme Garcês Pacheco Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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NEUTOXMER

“The greatest enemy of knowledge is not ignorance, it is the illusion of knowledge.”

Stephen Hawking

o júri / the jury

presidente

Professora Maria de Lourdes Gomes Pereira,
Professor Associado c/ Agregação

Doutora Joana Raimundo Pimenta,
Investigadora de Pós-Doutoramento do Instituto Português do Mar e da Atmosfera

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palavras-chave

Mercúrio inorgânico; Metilmercúrio; Neurotoxicidade; *Stress* Oxidativo; Cérebro; Peixe

resumo

O cérebro de peixes mostrou ser um órgão-alvo de várias formas orgânicas de mercúrio (Hg), principalmente metilmercúrio (MeHg). Pelo contrário, o conhecimento da neurotoxicidade do mercúrio divalente – Hg(II) – e a sua capacidade de acumulação em cérebro de peixes é muito escasso. A prevalência de informação sobre a neurotoxicidade de MeHg baseia-se, provavelmente, na percepção da sua elevada toxicidade, associada há sua rápida entrada no organismo e elevada distribuição. No entanto, foi também observado que as diferentes formas de Hg partilham a mesma forma tóxica e, por isso, a sua neurotoxicidade dependerá essencialmente da biodisponibilidade ambiental. De modo a contribuir para colmatar esta lacuna científica, realizaram-se 2 experiências com sargos juvenis (*Diplodus sargus*), que compreenderam períodos de exposição e pós-exposição, designadamente: experiência A – exposição via água a Hg(II) (2 µg L^{-1}); experiência B – exposição via alimento a MeHg (8,7 µg g^{-1}). Ambas as experiencias seguiram o mesmo desenho experimental, consistindo em 4 períodos de exposição (E) (dias 1, 3, 7 e 14) e 2 períodos de pós-exposição (PE) (dias 14 e 28). Foi mantido um grupo controlo em água do mar e ração não contaminada ao longo de toda a experiência. Em cada tempo de exposição e pós-exposição foram colhidos cérebros de *D. sargus* para determinação de Hg total (tHg) (experiência A), MeHg (experiência B) e parâmetros de *stress* oxidativo (ambas as experiências). O Hg(II) também foi quantificado no cérebro dos peixes expostos a MeHg. Embora em ambas as experiências o Hg total tenha atingido o seu máximo de acumulação ao dia 14 de exposição, os níveis maiores correspondem à exposição a MeHg (7,0 µg g^{-1} vs. 1,4 µg g^{-1} para Hg(II)). Os cérebros de peixes expostos a Hg(II) não eliminaram Hg, enquanto os níveis de MeHg diminuíram significativamente no período de pós-exposição (em média para 3,5 µg g^{-1}). Além disso, verificou-se uma reduzida ativação das defesas antioxidantes nos cérebros de peixes expostos a Hg(II), caracterizada principalmente pelo aumento das atividades de superóxido dismutase (SOD) e glutationa redutase (GR). A baixa proteção antioxidante (reforçada pela diminuição da atividade de GPx (glutationa peroxidase)) esteve provavelmente na base do dano oxidativo, tal como revelado pelo aumento dos grupos carbonilo (indicador de dano oxidativo em proteínas) ao longo dos períodos de exposição e pós-exposição. A exposição de *D. sargus* a MeHg conduziu a um cenário diferente, principalmente caracterizado pela ativação de defesas antioxidantes (SOD, catalase (CAT), GPx, glutationa S-transferase (GST), glutationa total (GSHt)) que conseguiram prevenir o dano oxidativo em lípidos e proteínas. Apesar de se ter registado uma maior acumulação de MeHg no cérebro de *D. sargus*, verificou-se uma maior vulnerabilidade do cérebro a Hg(II), tal como evidenciado pela ocorrência de dano oxidativo e pela menor resposta do sistema antioxidante. Globalmente, o Hg(II) revelou ter um maior potencial neurotóxico, o que aponta para a relevância de considerar esta forma de Hg, juntamente com o MeHg, em futuros estudos focados na saúde animal e humana.

keywords Inorganic mercury; Methylmercury; Neurotoxicity; Oxidative stress; Brain; Fish

abstract

Fish brain demonstrated to be a target organ for organic mercury forms (mainly methylmercury – MeHg). However, there is little information on the neurotoxicity of divalent mercury (Hg(II)) and its ability to accumulate in fish brain. The prevalent information on MeHg is likely based in the perception of its higher toxicity associated with rapid uptake and distribution. Nevertheless, it has been also stated that the different forms of Hg share the same toxic chemical entity and, thus, neurotoxicity depends mainly on the environmental bioavailability. To clarify this research gap, two experiments comprising exposure and post-exposure periods were performed with juveniles of white seabream (*Diplodus sargus*), namely: experiment A - waterborne exposure to Hg(II) (2 μg L^{-1}); experiment B - dietary exposure to MeHg (8.7 μg g^{-1}). Both experiments followed the same experimental design, consisting in 4 exposure periods (E) (days 1, 3, 7 and 14) and 2 post-exposure periods (PE) (days 14 and 28). A control group was kept throughout both experiments in clean seawater or fed with uncontaminated food. At each time, brain was collected for determination of total Hg (tHg) (Experiment A), MeHg (experiment B) and oxidative stress endpoints (both experiments). Though Hg accumulation reached maximum values in brain of both experiments after 14 days of exposure, the highest levels were reached upon exposure to MeHg (7.0 μg g^{-1} vs. 1.4 μg g^{-1} for $HgCl_2$). Interestingly, fish brain exposed to $HgCl_2$ was not able to eliminate Hg, while MeHg levels decreased significantly in the post-exposure period (to a mean of 3.5 μg g^{-1}). Moreover, there was a poor activation of antioxidant defenses in fish brain exposed to Hg(II), mainly characterized by increase of superoxide dismutase (SOD) and glutathione reductase (GR) activities. The low protection afforded by antioxidants (confirmed by glutathione peroxidase (GPx) activity decrease) was probably on the basis of oxidative damage, as revealed by the enhancement of protein carbonyl groups in exposure and post-exposure periods. MeHg accumulation led to a different scenario, mainly characterized by an activation of antioxidant defenses (SOD, catalase (CAT), GPx, glutathione S-transferase (GST), total glutathione content (GSHt)) that were able to prevent oxidative damage on proteins and lipids. Despite the higher accumulation of MeHg in fish brain, there was a higher vulnerability of fish brain to Hg(II), depicted in the occurrence of oxidative damage and less responsiveness of the antioxidant systems. Thus, Hg(II) revealed a higher neurotoxicity potential, pointing out the relevance to consider this Hg form, together with MeHg, in further studies concerning wildlife and human health.

Table of contents

1. INTRODUCTION

1.1. Mercury as a global threat and its occurrence in aquatic systems

Mercury (Hg) is one of the most hazardous contaminants that may be present in aquatic environments. For the European Water Framework Directive (WFD) it is considered a high priority pollutant, while UNEP (*United Nations Environment Programme*) described Hg as a global threat to human health and wildlife. Large amounts of mercury (mainly inorganic mercury) have been accumulated in the abiotic environment, particularly in surface soils and sediments, as a result of past releases. Nowadays, anthropogenic emissions of Hg to the environment are well restricted but contaminated soils and sediment still constitute a source of Hg to the aquatic systems. Much of the mercury released from artisanal and small-scale gold mining goes into rivers and lakes. Hg concentrations in streams and rivers near Hg deposits may contain up to 0.1 mg L^{-1} while near gold mining areas, levels of the total Hg in surrounding water can reach values from 0.0001 to 19.82 mg L^{-1} (Monteiro et al. 2010).Additionally, Hg continues to be used in pesticides and fungicides, and thus can reach aquatic systems also by this route. Finally, deforestation, especially in the Amazon Basin, can lead to extensive soil erosion and thus the release of Hg previously accumulated in the soils (Hacon et al. 2008).

Since 2005, the emissions of Hg to the environment have increased as a result of anthropogenic activities such as mining, fossil fuel combustion, industries of chloride and sodium hydroxide production through the electrolysis of brine and the production of electricity, measurement instruments (barometers), fluorescence tubes, alkaline batteries and usage in dental medicine; it is likely that emissions will be even higher in 2050 (UNEP 2013). Mercury is a global pollutant that can be find in places that did not ever received direct discharges, like the polar regions (Clarkson 2002). This is related with the Hg biogeochemical cycle displayed in figure 1. Particularly, Hg vapour can be oxidized in the air and returns to the earth's surface in rainwater settling in the sediment and being later released by resuspension or diffusion. Measurements made along the years show that atmospheric mercury has increased since the beginning of the industrialized period, associated with the increasing of population, urbanization and industrialization (Gupta et al. 2009), and thus, mercury pollution is often viewed as a global problem (Klaassen et al. 1986).

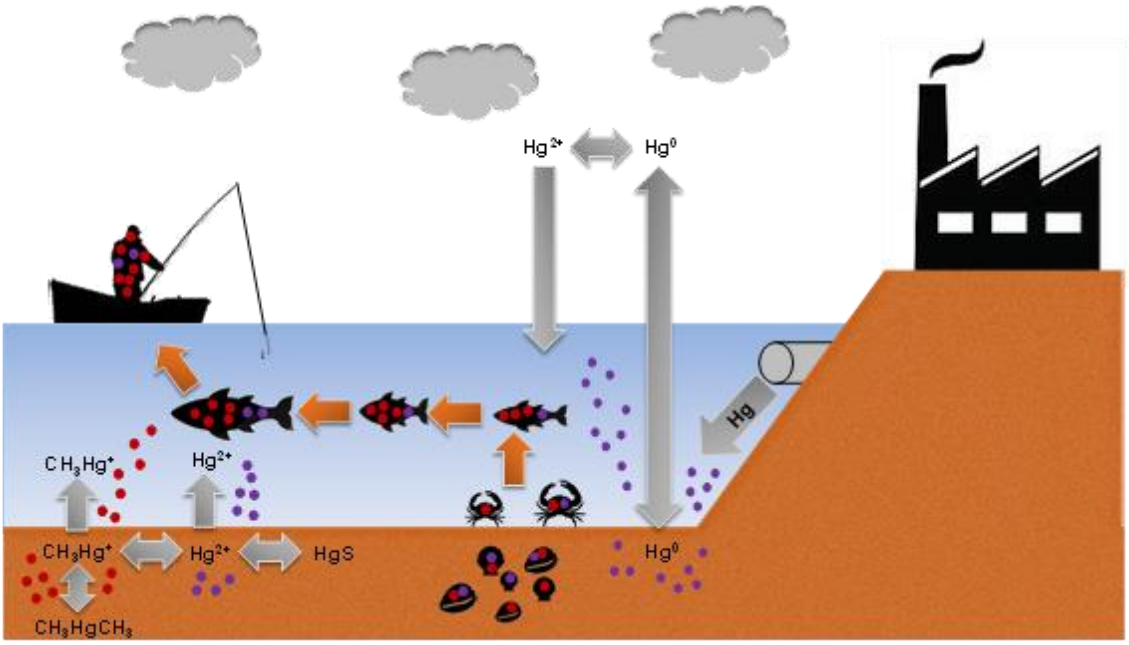


Figure 1 – Representative diagram of mercury cycle with inorganic and organic forms (purple and red, respectively). Adapted from Pereira 2014.

The presence of this element in aquatic environments can vary between two forms: organic (methyl or ethyl-mercury - MeHg or EtHg, respectively), where MeHg is the most prevalent, and inorganic compounds. Inorganic forms of mercury result from the binding of particulate ions as Hg^{2+} and Hg^{+} with elements such as chloride ($HgCl_2$), sulfur (HgS) or oxygen (HgO). The inorganic mercury is the most abundant form in the abiotic compartments, namely water and sediments. When inorganic Hg is reduced to elemental Hg, it can be re-emitted to the atmosphere by volatilization (Hudson et al. 1995) and when it binds to particulates in water, it can settle out rapidly and be accumulated in sediments (UNEP 2013).

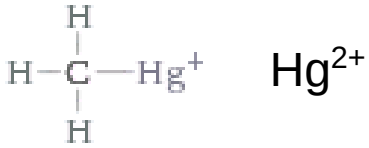
Inorganic mercury is transformed into MeHg through natural microbial processes (UNEP 2013), as specific bacteria that process sulfate (SO_4^{2}-) in the environment take up iHg, and through metabolic processes convert it to MeHg (River 1975). MeHg is mainly present in the sediment and ingested by zooplankton that filters the water and feed on algae, this way MeHg increases at each step in the aquatic food chain (Chen et al. 2012).

The exact mechanism(s) by which Hg enters the food chain remain(s) largely unknown, and probably varies among ecosystems (e.g. MeHg levels in freshwater fish can vary with the level of deposition of mercury from the air) (UNEP 2013). In contaminated environments, Hg stored in sediment (in the organic and inorganic forms) may be transferred to water and to benthic organisms (Linnik & Zubenko 2000). By trophic

transfer, Hg can easily reach fish, being thus bioaccumulated via the food chain (Wei et al. 2014), confirmed by the presence of higher Hg contents in predatory fish of higher trophic levels (Wei et al. 2014). In fish, most of the Hg occurs as MeHg and is therefore available for higher trophic levels (Lacoue-Labarthe et al. 2009). Both inorganic Hg and MeHg can be accumulated in fish organs. Thus, fish are considered for the understanding of toxicant uptake, accumulation and related biological responses, being hence crucial its employment for environmental health assessment of aquatic systems. In view of that, there are several works that used the accumulation and biological effects of Hg in fish as a mean to assess the environmental health status (Van der Oost et al. 2003; Fernandes et al. 2007; Pereira et al. 2009; Mieiro et al. 2010; Mieiro et al. 2011; Pereira et al. 2014).

1.2. Mercury interactions with biological systems and toxicity

Organic forms of Hg are considered the most toxic (Leong et al. 2001; Holmes et al. 2009) even though inorganic forms as Hg(II) are more abundant (Boening 2000). Several factors, such as molecular weight, charge, lipid solubility and membrane composition, affect membrane transport of chemicals (Manahan 1992). Hg(II) and MeHg in particularly present several differences in these aspects being the main one the presence of an extra $–CH_3$ group in MeHg as represented in figure 2 (National Research Council 2000).



(a) (b)

Figure 2 - Chemical composition of (a) MeHg and (b) Hg(II)

In the cells, the complex formed from the binding of MeHg and cysteine, MeHg-S-Cys, mimics the structure of methionine, a neutral amino acid. It is through this mimicry that MeHg is able to pass through the blood brain barrier and into nerve cells, where it impairs their function and exhibits toxic effects (Ni et al. 2011). The cellular uptake of Hg(II) occurs mainly by diffusion through the lipid membrane of lipid-soluble mercury complexes (Morel et al. 1998). The diffusion of Hg species involves mainly neutral species, even though

ionized species can pass through axonal transport (Rouleau et al. 1999). The high permeability of Hg(II) through lipid bilayer membranes is due to permeation of the neutral dichloride complex, $HgCl_2$ (Gutknecht 1981). The chemical bonding of the dichloro with the mercuric complex, $HgCl_2$, forms the uncharged complex that diffuses rapidly through lipid bilayers, leading to an efficient cellular uptake of mercury (Morel et al. 1998).

The presence of MeHg in higher levels of the food chain is justified by the lipid solubility, allowing its accumulation in the fatty tissue and mainly in muscle tissue (Morel et al. 1998), MeHg also represents most of the Hg form accumulated in fish (Hites 2004). Morel et al. (1998) also stated that the presence of lower levels of Hg(II) is also explained by the very low uptake rate in the intestinal wall in fish. It also has been shown that MeHg uses the interactions with multi-lamellar vesicles as dipalmitoyl, phosphatidylcholine, phosphatidic acid, phosphatidylglycerol and phosphatidylserine to enter the cell. MeHg is capable of binding itself to three molecules per lipid, inducing limited perturbations and loss of membrane integrity (Girault et al. 1997).

Scientific data show that Hg is cytotoxic, mutagenic, carcinogenic, and affects a wide variety of tissues and organs, being primarily known for its neurotoxic properties (Wiener et al. 2003; UNEP 2013). Its presence in the global environment is considerably increased by anthropogenic activities (Hutcheson et al. 2014), and despite being considered to have higher toxicity in organic forms, Hg is overall more toxic than any other nonradioactive element (Korbas et al. 2013). Some of the reported Hg effects in fish are inhibition of hepatic biotransformation enzymes (Guilherme et al. 2008a), oxidative stress in brain (Berntssen et al. 2003), genotoxicity in blood (Guilherme et al. 2008b) and reproductive alterations (Crump & Trudeau 2009).

Each Hg form has an individual toxicological profile, metabolic fate and biochemical effect. Although MeHg has a higher toxicity level, Hg(II) is the most available form of Hg in the aquatic environment (Monteiro et al. 2010; Depew et al. 2012). Hg(II) is able to impair the protein and lipid membrane functions (Monteiro et al. 2010), while MeHg effects vary from mortality (Eto 1997) to oxidative stress (Drevnick et al. 2008; Schwindt et al. 2008; Mieiro et al. 2010) and changes in gene transcription (Moran et al. 2007).

1.3. Fish as biosentinels of aquatic contamination and models in neurotoxicology

Fish play a major role in aquatic food-webs, occupying different habitats in the same ecosystem and have different feeding behaviours. Thus, the understanding of toxicant uptake, kinetic and responses in fish may have a high ecological relevance (Van der Oost et al. 2003). Despite the limitation associated to their mobility, fish are generally considered to be the most feasible organisms for pollution monitoring in aquatic systems (Van der Oost et al. 2003). In fact, international monitoring protocols include measurements in fish species (WHO 1993). Fish have already been used as models in neurotoxicity studies, showing behaviour alterations, pathological damage and lipid peroxidative damage in brain of *Salmo salar* upon exposure to MeHg (Berntssen et al. 2003), Hg-induced breakdown of redox-defense system in *Liza aurata* (Mieiro et al. 2010) and potential harmful accumulation of Hg in brain of *Dicentrarchus labrax* (Mieiro et al. 2011). Parng et al. (2007) also demonstrated that neurotoxicity can be assessed in *Danio rerio* optic nerves, motor neurons, dopaminergic neurons, showing susceptibility to some neurotoxins as ethanol and retinoic acid. Taking all this into considerations, fish can be considered suitable models in neurotoxicity studies.

Figure 3 – *Diplodus sargus* (*Diplodus sargus sargus*, White seabream)
Source: http://www.fishbase.org/[1]

Sparidae species have been largely used in the environmental health assessment mainly because they are a commercialized and native fish family, susceptible to biological invasions such as green alga *Caulerpa racemosa* or products from anthropogenic

[1] Available at: http://www.fishbase.org/summary/1753, September 2015.

activities (Gorbi et al. 2014). In fact, white seabream has potential for aquaculture diversification due to its high market value, easy adaptation to captivity (Abellán & Basurco 1999). This species has an important role in toxicological studies such as Hg exposure (Pereira et al. 2015), oxidative status after supplemented diets (Enes et al. 2012) and even assesses *Diplodus sargus* eggs tolerance to surface disinfectants, since it represents species of interest for Mediterranean aquaculture (Katharios et al. 2007).

The white seabream *D. sargus* was selected as a test organism in the present thesis since it is an abundant species in estuarine systems that can still present high levels of contamination, including of Hg (Pereira et al. 2011). In this context, *D. sargus* was previously employed as a model species to investigate the toxicokinetics of iHg (Pereira et al. 2015). Moreover, *D. sargus* is effortlessly maintained in the laboratory and aquaculture and it is easy to handle.

1.4. Toxicokinetics of mercury in fish and the brain as a target organ

The kinetics of a xenobiotic is the quantification of the time course in the body during the processes of absorption, distribution, biotransformation, and excretion or clearance (Manahan 1992). The assessment of Hg toxicity is determined by various factors such the Hg assimilation efficiency, aqueous or food uptake-rate and elimination-rate, taking into account that accumulation rate depends on the assimilation efficiency (Wang & Wong 2003). It is well known that Hg accumulation in fish can occur through different pathways, namely directly from water, via uptake from suspended particles and sediment, or by the consumption of lower trophic level organisms (Watras et al. 1998).

It is well established that some of the mechanisms present in the cellular environment render the mercury ion ineffective in disturbing the normal biochemical processes of the cell: efflux pumps that remove the ion from the cell; enzymatic reduction of the metal to the less toxic elemental form; chelation by enzymatic polymers; binding mercury to cell surfaces; precipitation of insoluble inorganic complexes (usually sulfides and oxides), at the cell surface and biomethylation by bacteria with subsequent transport through the cell membrane by diffusion (Boening 2000).

Mercury absorption by fish involves their transfer to the blood through the epithelial barrier of gills, digestive organs or directly into the muscle (Jezierska & Witeska 2006; Régine et al. 2006; de Oliveira Ribeiro et al. 2008; Lacoue-Labarthe et al. 2009; Ibrahim 2015). This distribution is dependent on uptake route (food *versus* water) and species of Hg taken up (e.g. MeHg *versus* Hg(II)). However, Rouleau et al. (1999) found similar

levels of accumulated Hg in gills, kidney, skin, liver parenchyma and blood after exposure of brown and rainbow trout to Hg(II), excepting hepatic blood vessels that presented higher levels.

Gills are the main organ of trace elements uptake, such as Fe and Hg, in fish (Karan et al. 1998; Dalzell & Macfarlane 1999), since they are directly exposed to water, providing a wide uptake surface area. Dissolved Hg alters the permeability characteristics of gills, increasing passive ionic effluxes (Lock et al. 1981); this process occurs mainly through the uptake of Hg(II) instead of Ca^{2+} (Klinck et al. 2005). Particularly, it was found in *Gambusia holbrooki* that the accumulation following 14 days Hg(II) exposure induced morphological modifications, such as the thickening of primary lamellar epithelium (Jagoe et al. 1996). The MeHg uptake mainly depends on its speciation, forming complexes with the ligands OH- and Cl- (Major et al. 1991). Block et al. (1997) also demonstrated that MeHg exposure via water is mainly taken up across the apical membrane of the gill epithelial cells of *Phoxinus phoxinu* where is mostly accumulated. The high renewal rate of branchial epithelium triggered by constant exfoliation and erosion, counteracted by an intense cell division rate (Potter et al. 1997), are factors that contribute to the assessment of recent or current exposures through gills (Pereira et al. 2010).

Some studies showed that neurosensory organs, such as eyes (Korbas et al. 2013; Pereira et al. 2014), are also susceptible to Hg accumulation and toxicity, being also a potential uptake route, even though they are protected by the BRB (blood retinal barrier). Pereira et al. (2015) showed that Hg(II) can be distributed through the blood to eye appearing to be the preferential uptake route. MeHg can also cross the BRB due to its high affinity with the sulfhydryl groups of organic molecules, passing through organic barriers by connecting to molecules present in the organism, such as cysteines (Quig 1998).

The liver has also high affinity with Hg but it can also excrete Hg(II) into the faeces, as a result of biliary secretion. It also has other hepatic defence mechanisms such as glutathione and MTs (Wei et al. 2014) that allow the Hg excretion or toxicity decrease. The bile also functions as a backup when exhaustion of the detoxification strategies occurs (Pereira et al. 2015). MeHg also has the capability of transport across the liver, forming a glutathione-MeHg complex, which has been detected in hepatic tissue and bile (Dutczak & Ballatori 1994).

Mercury reaches the brain mainly via bloodstream in both Hg(II) contaminated water, after demethylation in liver (Pereira et al. 2015) and MeHg contaminated food (Wiener &

Spry 1996; Watras et al. 1998). There are more studies focused on the neurotoxicity of MeHg than on Hg(II). However, it has been also stated that the different forms of Hg share the same toxic chemical entity and, thus, neurotoxicity depends mainly on the external bioavailability (De Flora et al. 1994). Several other studies documented the occurrence of Hg(II) (Rouleau et al. 1999; Rouleau et al. 2003; Mieiro et al. 2010; Mieiro et al. 2011; Farina et al. 2013; Pereira et al. 2014; Pereira et al. 2015) and MeHg (Wang & Wong 2003; Mieiro et al. 2010; Mieiro et al. 2011; Branco et al. 2012; Depew et al. 2012; Farina et al. 2013; Jesus et al. 2013; Pereira et al. 2014) in brain of fish.

The brain has distinct accumulation capacity where the thiols and MTs cysteine-rich intracellular proteins are important ligands for Hg(II) in central nervous system (CNS). Hg(II) can only reach the brain after crossing the BBB (blood brain barrier) but its occurrence in the brain was also previously attributed to MeHg uptake and subsequent demethylation (Korbas et al. 2010). Despite Hg(II) could cross BBB bi-directionally, by membrane carrier systems, Hg(II) influx and efflux from brain can lead to its accumulation in brain over time (Pereira et al. 2015). It is possible that the Hg(II) is the proximate toxic agent responsible for the brain damage (Clarkson 2002), since appeared to be more toxic than MeHg to glial cells and neurons in immature aggregate cultures of rat telencephalon (Monnet-Tschudi et al. 1996). Other mechanisms can also support the distribution of water Hg(II) exposure, where Hg(II) can reach the brain via axonal transport, avoiding this way the BBB and being able to persist for years in nerve cells. Prime candidates for the transport of waterborne Hg(II) are sensory nerves innervating from water-exposed sensory organs, followed by axonal transport along neurons toward their termination sites in rhombencephalon and cerebellum (Rouleau et al. 1999). Hg(II) accumulation in brain could induce alterations in the nervous system, endangering the fish, since the search for food, recognition of predators, communication, and orientation could be compromised (Rouleau et al. 1999).

The efflux of MeHg through brain capillary endothelial cells of BBB was already proved to occur in association with glutathione, as reported in other cell systems and previously described for manganese and iron (Yokel 2009). MeHg is present in the body as water-soluble complexes if not attached to the sulfur atom of thiol ligands. It enters the endothelial cells of the BBB as a complex with L-cysteine (Clarkson 2002) and the plasma MeHg-glutathione complex serves as a source of MeHg-cysteine (Kerper et al. 1992). Several thiol-containing complexing agents have been successfully used in humans to reduce the MeHg in blood (Clarkson et al. 1981).

At the light of this controversy, more research is needed to evaluate the neurotoxicity

of Hg(II) and MeHg in fish brain, since it has a crucial role in fish fitness and survival (Pereira et al. 2015).

1.5. Oxidative stress involvement on Hg neurotoxicity in fish

Although neurotoxicity mechanisms of Hg is still unclear, especially in fish (Mieiro et al. 2011), it was previously associated with the occurrence of oxidative stress and the formation of reactive oxygen species (ROS) (Berntssen et al. 2003; Mieiro et al. 2010 and 2011; Pereira et al. 2014), due to the binding of mercury to GSH or cysteine, promoting the accumulation of reactive oxygen species (ROS), since they would usually be eliminated by GSH (Sarafian 1999). It is well established that oxidative stress is a key pathway to trigger Hg neurotoxicity in mammals (Aschner & Aschner 2007; Farina et al. 2013) that results from the imbalance between the production and removal of ROS (Ercal et al. 2001).

Antioxidants play an extremely important role in maintaining cell homeostasis, and when their activity is adversely altered, the formation of ROS can lead to oxidative damage expressed by lipid peroxidation of the cellular membranes, enzymatic inactivation and cell aging, and DNA damage (Stohs & Bagchi 1995; Hirata et al. 2004; Guilherme et al. 2008b). This would eventually lead to an enzymatic response in the cytosol designed to decrease the rate of oxidative damage (Berntssen et al. 2003; Stringari et al. 2008; Vieira et al. 2009; Huang et al. 2010; Oliveira et al. 2010). To prevent damage caused by ROS, cells usually react by increasing the levels of protective antioxidant enzymes and non-enzymatic free radical scavengers like reduced glutathione (GSH).

The most relevant antioxidants involved in ROS elimination are described below, as well as their respective role. Additionally, the schematic interaction between antioxidants and ROS is presented in figure 4. Mercury is highly reactive with sulphydryl groups of proteins, forming covalent bonds with reduced glutathione (GSH) and cysteine residues of proteins. Moreover, GSH directly binds to MeHg, causing their irreversible excretion (Ballatori & Clarkson 1982; Quig 1998). The conjugate MeHg-GSH diminishing the intensity of the antioxidant response due to the lack of GSH, resulting in greater activity of the free Hg ions disturbing GSH metabolism and damaging cells (Franco et al. 2009). GSH has also a role as cofactor of other enzymes such as GST and GPx (Cnubben et al. 2001). Thus, it has been noted that MeHg promotes a decrease in intracellular GSH levels, which is considered one of its cytotoxic effects (Yee & Choi 1996), since ROS are no longer eliminated by GSH cells (Franco et al. 2009) leading to the activation of other

antioxidant defenses such as CAT, SOD, GPx, GST and GR. Additionally, the inhibition of antioxidant enzymes has been referred as a relevant mechanism involved in oxidative stress due to Hg (Roos et al. 2009). Only a few studies have searched for the modulation of antioxidant enzymes and alterations in GSH content in fish brain after Hg exposure (Berntssen et al. 2003; Mieiro et al. 2011).

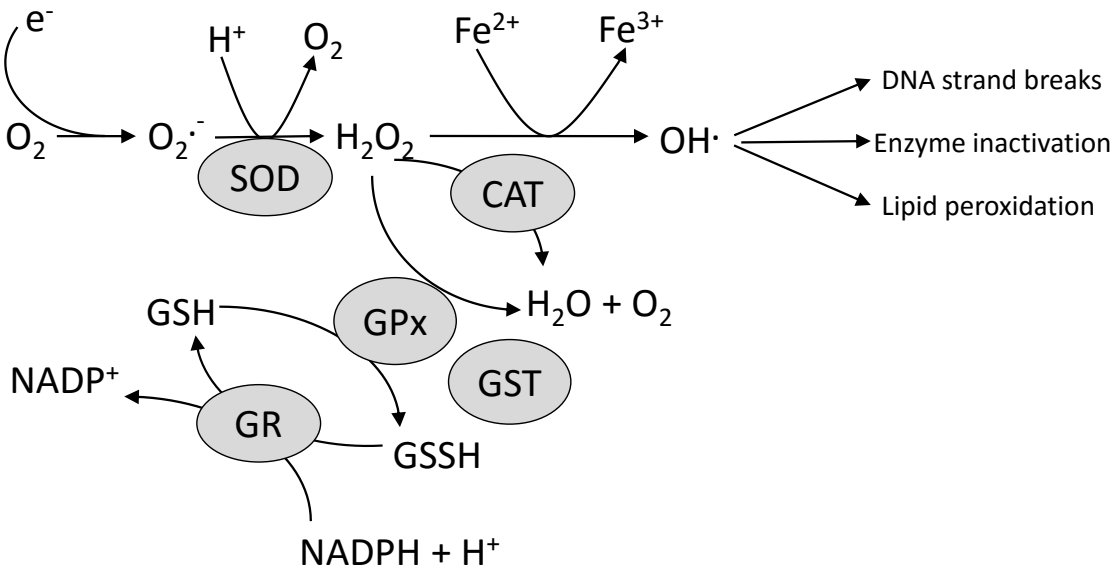


Figure 4 – Graphical representation of the relations between the main antioxidants enzymes and ROS (Adapted from Macdonald 2003).

- Superoxide dismutase (SOD): catalyzes the dismutation of the superoxide ion (O_2●) in hydrogen peroxide (H_2O_2) (Peskin & Winterbourn 2000);
- Catalase (CAT): degrades hydrogen peroxide into water and oxygen molecules (Rojkind et al. 2002);
- Glutathione reductase (GR): catalyses the transformation of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$. Therefore, GR maintains the GSH/GSSG balance under oxidative stress conditions (Meister 1983);
- Glutathione peroxidase (GPx): detoxifies organic and inorganic peroxides, by using GSH as a cofactor. GPx is an integral part of the mechanisms that contribute to diminish the rate of lipid peroxidation (Epp et al. 1983);
- Glutathione S-transferase (GST): Metabolizes several xenobiotic compounds and is responsible for conjugating electrophilic compounds with GSH for the purpose of detoxification, and may also play an important role in deactivating the products of lipid peroxidation and their derivatives (Cnubben et al. 2001).

Mercury contamination also induces lipid peroxidation (LPO) and carbonyl groups formation. LPO consists in the oxidative degradation of lipids, in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage (Muller et al. 2007), while carbonyl groups (aldehydes and ketones) is the most general indicator and commonly used as a measure of protein oxidation (Dalle-Donne et al. 2003). Carbonyl groups determinations have been used effectively in eel (Almroth et al. 2005), brown trout (Almroth et al. 2008) and bloch (Parvez & Raisuddin 2005).

Reactive oxygen species are known to convert amino groups of proteins altering protein structure or function. Carbonyl groups can be introduced in proteins by different pathways, predominantly via metal catalysed oxidation. Carbonyl groups can also increase via adduction carbonyl-containing oxidized lipids, sugars containing carbonyls (Requena et al. 2003), oxidation of protein side chains (proline, arginine, lysine, and threonine), oxidative cleavage of proteins by oxidation of glutamyl side chains (Dalle-Donne et al. 2003). They can also be introduced into proteins by secondary reaction of the nucleophilic side chains of cysteine, histidine, and lysine residues, with aldehydes produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) (Dalle-Donne et al. 2003). The increase of carbonyl groups content correlates well with protein damage caused by oxidative stress (Shacter et al. 1994). The formation of carbonyl derivatives is non-reversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting in breakdown of proteins by proteases due to increased susceptibility (Almroth et al. 2005).

There is still a lack of data concerning carbonyl groups in brain of fish after exposure to Hg (both Hg(II) and MeHg). However, the content of carbonyl groups increased in gills, kidney and liver of *Clarias gariepinus* (African catfish) upon exposure to Hg(II) (Ibrahim 2015).

1.6. Thesis motivation, outline and objectives

Still, there is a lack of studies that can elucidate about the modulation of the antioxidant system and subsequent emergence of oxidative damage in fish brain after exposure to Hg(II) and MeHg. Moreover, the neurotoxicity of Hg(II) remains completely unclear. For instance, Berntssen et al. (2003) found a significant increase of lipid peroxidative products after dietary exposure to MeHg together with a decrease of

antioxidant enzyme activity (SOD and GPx), while no significant changes of those endpoints were observed upon exposure to iHg in food. Hg(II) seems to be accumulated in brain as HgSe that is an inert complex (Korbas et al. 2010). Despite that, neurotoxicological effects were largely reported at different levels of complexity after exposure of rats and fish to $HgCl_2$. Moreover, the Berntssen et al. (2003) study compared the neurotoxicity of MeHg and Hg(II) but missed the time-evolution of Hg accumulation and oxidative stress responses, as well as the evaluation of Hg depuration and the reversibility of oxidative stress events. The current thesis aimed to fill that knowledge gap.

Another original aspect of this thesis is the assessment of Hg-induced neurotoxicity by the evaluation of protein carbonyls. The susceptibility of the brain to the formation of carbonyl groups was already demonstrated but never in association with Hg exposure. LPO is the most used damage marker to determine mercury-induced neurotoxicity (Mieiro et al. 2011) but other studies have also shown susceptibility of the brain to the formation of carbonyl groups in *Boleophthalmus boddarti* (Ching et al. 2009), *Clarias batrachus* (Maiti et al. 2010) and *Danio rerio* (Tseng et al. 2011). The use of carbonyl groups as damage indicator present some advantages, in comparison with LPO, the oxidised proteins are relatively stable and oxidised proteins can be degraded within hours and days, whereas lipid peroxidation products can be detoxified within minutes (Dalle-Donne et al. 2003).

Thus, this study compares for the first time the impact of Hg(II) and MeHg in brain of fish (white seabream - *Diplodus sargus*) by the combination of accumulation levels and oxidative stress profiles in a time-evolution perspective. For that purpose, two experiments were performed, both comprising exposure and post-exposure periods, namely: experiment A consisting in the waterborne exposure to $HgCl_2$ (2 µg L^{-1}); experiment B consisting in the dietary exposure to MeHg (8.7 µg g^{-1}). Realistic levels of Hg both in water and in food were tested in order to produce reliable data to environmental health assessment. Fish brain was surveyed after 1, 3, 7 and 14 days of Hg exposure (Hg(II) and MeHg), as well as following a post-exposure period of 14 and 28 days, for evaluation of Hg accumulation levels, antioxidant enzymes and oxidative damage indicators. Hence, the specific aims of this thesis were the following:

1. to assess the accumulation of Hg(II) and MeHg in *D. sargus* brain following exposure to contaminated water and food, respectively;
2. to clarify the elimination of Hg(II) and MeHg from *D. sargus* brain during depuration;

3. to clarify the potential of Hg(II) and MeHg to alter the oxidative stress status in brain of *D. sargus* by the assessment of antioxidant defenses;
4. to assess the neurotoxic potential of Hg(II) and MeHg in *D. sargus* brain by the evaluation of damage in lipids and proteins;
5. to establish a causal relationship between levels of Hg(II) and MeHg in brain and the previous responses.

Since oxidative stress is a chief event for the neurotoxicity of Hg, this thesis can provide relevant insights for the neurotoxicology field. One of the main impacts of this thesis can be related with the clarification of the neurotoxic potential of Hg(II). After accumulation of Hg(II) and MeHg in brain, both forms can lead to alterations in the cellular protection against oxidative stress, leading eventually to damage of lipids and proteins. Such repercussions may eventually compromise fish neurosensory performance and survival.

2. MATERIAL AND METHODS

2.1. Experimental set-up sampling

Two experiments, with the same design, were performed with juvenile white seabreams (*Diplodus sargus*) provided by an Aquaculture Research Station (IPMA - Olhão, Portugal) (weight: 130 ± 13 g; total length: 18 ± 1 cm) (Figure 5a and 5b). Fish were held in 300 L fiberglass tanks in an average initial density of 0.012 kg L^{-1}, under a 10:14 light: dark photoperiod. Experiment A concerned the exposure of fish to $HgCl_2$ [divalent Hg - Hg(II)] via water contamination (Figure 5a), while in experiment B fish were exposed to MeHg through contaminated pellets (Figure 5). In both experiments, seawater was renewed daily (around 80%) and fish were fed once a day, namely 1-2 hours before water renewal. In all sampling days, fish were not fed in the 12 hours preceding fish handling. Water temperature, salinity and pH were monitored daily throughout both experiments, varying as follows, respectively: 13.5 ± 0.3 ºC, 35 ± 2 and 7-8.

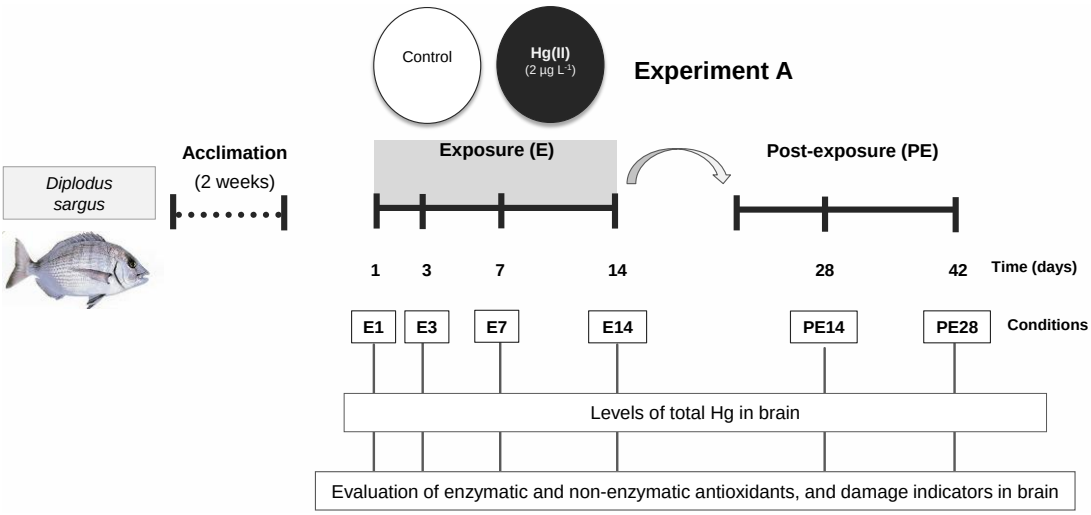


Figure 5a – Design of experiments with white seabream (*Diplodus sargus*) comprising Hg(II) exposure (2 µg L^{-1}) (Experiment A and Experiment B, respectively). Prior to Hg exposure, fish were allowed to acclimatize for 2 weeks (T0). Fish were exposed for 1, 3, 7 and 14 days (conditions E1, E3, E7 and E14, respectively). Thereafter, fish were transferred to clean water and allowed to recover for 14 and 28 days (PE14 and PE28 conditions, respectively). In parallel, control groups were also considered.

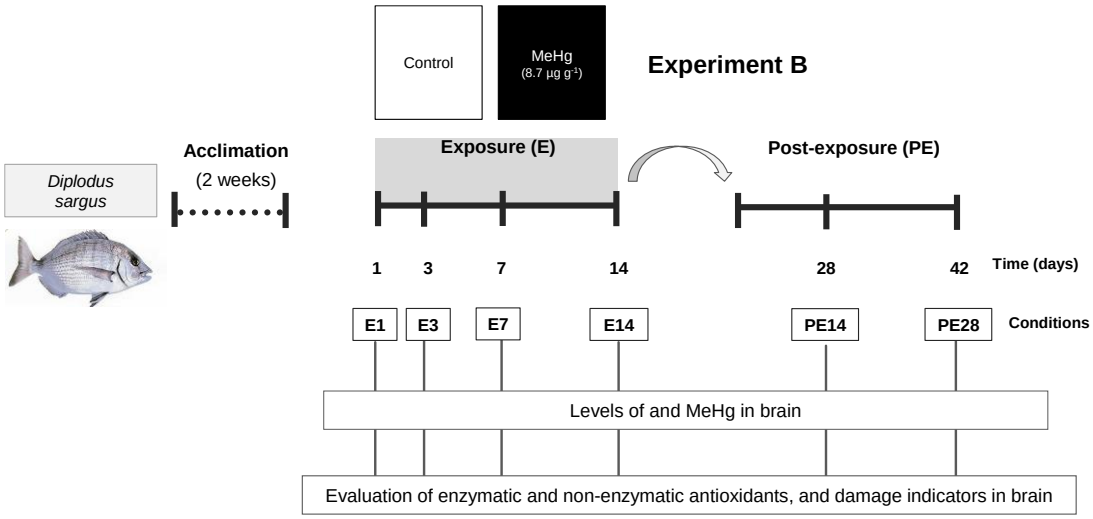


Figure 5b – Design of experiments with white seabream (*Diplodus sargus*) comprising Hg(II) exposure MeHg (8.7 µg g^{-1}) (Experiment B). Prior to Hg exposure, fish were allowed to acclimatize for 2 weeks (T0). Fish were exposed for 1, 3, 7 and 14 days (conditions E1, E3, E7 and E14, respectively). Thereafter, fish were transferred to clean water and allowed to recover for 14 and 28 days (PE14 and PE28 conditions, respectively). In parallel, control groups were also considered (continuation).

In experiment A, fish were fed with a commercial dry food [standard 3 mm from Sorgal (Portugal)] with total Hg levels lower than 0.01 µg g^{-1}. In exposure tanks, $HgCl_2$ (Sigma Aldrich) was added to the water in an aqueous solution in order to reach a final concentration of 2 µg L^{-1}. Mercury chloride was added on a daily basis after water renewal (i.e. daily water recontamination) during the exposure period. This divalent Hg level was established considering previous studies in contaminated areas (Horvat et al., 2003; Li et al., 2009) in order to mimic environmentally realistic conditions in contaminated areas. Control fish were kept throughout the experiment in tanks filled with clean seawater.

In experiment B, contaminated pellets by MeHg (8.7±0.5 µg g^{-1} dry weight) were used to feed fish of exposure tanks [3 mm pellets]. Natural food of *D. sargus* (i.e. *Nereis diversicolor*) from contaminated areas could have such levels of MeHg (Pereira et al. unpublished data). Control fish were fed with food prepared in the same occasion but without adding MeHg (MeHg levels lower than 0.01 µg g^{-1}).

Fish wellbeing deserved a permanent attention, in accordance with national and international guidelines for the protection of animal welfare.

In both experiments, fish were allowed to acclimatize to experimental conditions and routines for two weeks prior to Hg exposure. Eight fish for Hg determinations and another eight for oxidative stress analyses were sacrificed at each sampling time of both experiments (Figure 5a and 5b). The number of sacrificed fish was maintained for every sampling time, at each condition (n=8).

Both experiments followed the same experimental set-up, namely fish were exposed to divalent Hg and MeHg for 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days, in experiments A and B respectively. Thereafter, fish were transferred to clean water (post-exposure in exp. A) or fed with uncontaminated pellets (exp. B) and allowed to recover for 14 (PE14) and 28 days (PE28) (Figure 5). The experiment had a total duration of 42 days.

During the exposure period (at days 1, 3, 7 and 14) of experiment A, water samples were collected in triplicates from exposure and control tanks 24 hours after recontamination to quantify total Hg (tHg) levels, in order to prove that fish were subjected to the toxicant. Values of tHg in the exposure tanks varied between 0.05 and 0.36 μg L^{-1}, which would probably correspond to the minimum exposure concentration. Levels of tHg in the control tanks were below the detection limit throughout the experiment (0.1 ng L^{-1}). Identically, at days 28 and 42 (post-exposure period), both in control and in previously contaminated tanks, tHg was below the analytical detection limit.

2.2. Brain sampling

Immediately after collection, fish were anesthetized with tricaine methanesulfonate (MS-222), weighed, measured, and sacrificed by cervical transection. Fish were properly bled from the cardinal vein, using heparinised Pasteur pipettes, and then brain was removed and stored at -80 ºC until further processing for Hg determinations and evaluation of oxidative stress related endpoints.

2.3. Mercury analyses in brain

Brain samples from experiment A were firstly lyophilized, homogenized and then analysed for tHg by atomic absorption spectrometry (AAS) with thermal decomposition following by gold amalgamation in a Hg analyser (AMA) LECO 254 (Costley et al. 2000). Certified reference materials (Fish protein - DORM-3, Dogfish Liver Tissue - DOLT-4) from the Canadian National Research Council were used to ensure the accuracy of the procedure and the obtained values were consistent with the certified ones. In the current work, tHg levels in brain allowed interpretations on Hg(II) toxicokinetics based on the assumption that fish were exposed to Hg(II) and that no methylation was so far reported to occur in fish.

Brain samples from experiment B were processed and analyzed for MeHg in the Center for Analytical Research on the Environment (CARE) at Acadia University. Dried and homogenized samples were weighed using a Sartitorius ultra balance, approximately

10 mg of homogenate was transferred to a 2 mL polypropylene vial for MeHg analysis. Analytical procedure for MeHg analysis involved an alkaline digestion, ethylation, and purge and trap gas chromatography – atomic fluorescence spectrometry (Liang et al. 1994; Edmonds et al. 2012). Quality control methods included deionized water method blanks, sample replicates, analytical replicates and a comparative analysis with certified standard reference materials (DORM-3 and DOLT-4). All samples were blank corrected. Sample replicates were within accepted norms for MeHg (mean % RSD = 5.8 %, n=18). The certified reference materials showed excellent recoveries for MeHg. This technique has been used in previously published work (Edmonds et al. 2012).

2.4. Analyses of antioxidants and damage indicators in the brain

Tissue samples were homogenized in a 1:8 ratio, using a Potter-Elvehjem homogenizer, in chilled phosphate buffer (0.1 M, pH 7.4) (1 g of tissue/7 mL buffer). This homogenate was then divided in two aliquots, one for posterior lipid peroxidation (LPO) evaluation and another for post-microsomal supernatant (PMS) preparation. The aliquot for LPO determination of Hg(II) brain samples was stored with 10 µl butylated hydroxytoluene (BHT; prepared in 4% of methanol), 90 µl phosphate buffer plus 100 µl of homogenate while the LPO aliquot of MeHg brain samples was stored with 10 µl of BHT plus 100 µl of homogenate without the phosphate buffer. The PMS fraction was obtained by centrifugation in a refrigerated centrifuge (Eppendorf 5415R) at 13,400 g for 25 minutes at 4 ºC. Aliquots of PMS were divided in microtubes and stored at -80 ºC until spectrophotometric analyses at 25 ºC, which consisted on the following procedures:

- catalase (CAT) activity was assayed in PMS by Claiborne (1985) method as described by (Giri et al. 1996). Briefly, the assay mixture consisted of 1.95 mL phosphate buffer (0.05 M, pH 7.0), 1 mL hydrogen peroxide (0.030 M) and 50 µL of sample in final volume of 3 mL. Change in absorbance was recorded at 240 nm in a spectrophotometer (Jasco UV/VIS, V-530) and CAT activity was calculated in terms of µmol H_2O_2 consumed min^{-1} $mg^{-1}$of protein using a molar extinction coefficient (ε) of 43.5 $M^{-1}cm^{-1}$;
- superoxide dismutase (SOD) activity was measured in PMS using a spectrophotometric enzymatic kit (RANSOD TM, Randox). This methodology employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. Changes in absorbance were recorded in 30-second cycles for 3.5 minutes at 505 nm in a spectrophotometer (Jasco UV/VIS, V-530), and

SOD activity was calculated in terms of the percentage of inhibition of the reaction. One unit of SOD is the amount that causes a 50% inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD units per $mg^{-1}$of protein;

- glutathione peroxidase (GPx) activity was determined in PMS according to the method described by (Mohandas et al. 1984). The assay mixture consisted of 0.72 mL phosphate buffer (0.05 M, pH 7.0), 0.05 mL EDTA (1 mM), 0.05 mL sodium azide (1 mM), 0.025 mL GR (1 IU/mL), 0.05 mL reduced glutathione (GSH; 4 mM), 0.05 mL NADPH (0.8 mM), 0.005 mL H_2O_2 (0.5 mM) and 0.05 mL of PMS in a total volume of 1 mL. GPx activity was determined using a spectrophotometer (Jasco UV/VIS, V-530) by monitoring the oxidation of NADPH to $NADP^+$, resulting in an absorbance decrease at 340 nm. The absorbance was read every 30 seconds for a period of 3 minutes. GPx activity was calculated in terms of nmol NADPH oxidized $min^{-1}mg^{-1}$of protein using a ε of 6.22 $mM^{-1}cm^{-1}$;
- glutathione reductase (GR) activity was assayed by the method of (Cribb et al. 1989) with some modifications. Briefly, the assay mixture contained 0.025 mL of PMS fraction and 0.975 mL of NADPH (0.2 mM), glutathione disulfide (GSSG - 1 mM) and diethylene triaminepentaacetic acid (DTPA - 0.5 mM). The enzyme activity was quantified using a spectrophotometer (Jasco UV/VIS, V-530) by measuring the disappearance of NADPH at 340 nm during 3 minutes. The enzyme activity was calculated as nmol NADPH oxidized $min^{-1}mg^{-1}$of protein using a ε of 6.22 $mM^{-1}cm^{-1}$;
- glutathione-S-transferase (GST) activity was determined according to the method of (Habig et al. 1974) using CDNB (1-chloro-2,4-dinitrobenzene) as substrate. The assay was carried out in a 96-well microtiter plate with a 100 µL of PMS (previously diluted 1:65) and 175 µL of GSH (1.765 mM; prepared in phosphate buffer 0.2 M, pH 7.9). The reaction was initiated by addition of 30 µL of 1-chloro-2,4-dinitrobenzene (CDNB; 10 mM), and the increase in absorbance was recorded spectrophotometrically at 340 nm, during 5 min each 30 s. GST activity was expressed as nanomoles of thioether produced/min/mg of protein (ε=9.6 $mM^{-1}cm^{-1}$).
- total glutathione (GSHt) content was measured following the method of (Baker et al. 1990) adapted to a microplate reader by (Vandeputte et al. 1994). Protein content in the PMS was precipitated with trichloroacetic acid (TCA; 12%) for 1 hour

and then centrifuged at 12,000 g for 5 minutes at 4 ºC. GSHt was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5´-dithiobis-2-nitrobenzoic acid, Ellman's reagent) producing a yellow coloured 5-thio-2-nitrobenzoicacid (TNB). Reaction mixture containing 1 mM DTNB, 0.34 mM NADPH dissolved in a stock sodium phosphate buffer (143 mM with 6.3 mM EDTA, pH 7.4) was added to wells containing 40 µL of deproteinated PMS (previously diluted 1:3) and the reaction was started by adding 40 µL of 8.5 IU mL^{-1} GR. Formation of TNB was monitored by spectrophotometry at 415 nm, for 7 minutes using a SpectraMax 190 microplate reader. The results were expressed as nmol TNB conjugated min^{-1} mg^{-1} of protein (ε=14.1 mM^{-1} cm^{-1}).

- LPO was determined in the previously prepared homogenate as adapted by (Filho et al. 2001) after Bird and Draper (1984). Briefly, 1 mL of TCA (12 %) in aqueous solution, 0.90 mL of Tris–HCl (60 mM, pH 7.4, and 0.1 mM DTPA) and 1 mL of TBA (0.73 %) were added to 0.09 mL of the homogenate and well mixed. This mixture was heated for 1 h in a water bath set at boiling temperature and then cooled to room temperature, decanted into 2-mL microtubes and centrifuged at 15,800 g for 5 minutes. Absorbance was measured at 535 nm, and LPO was expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) formed per milligram of protein (ε=1.56×10^5 M^{-1} cm^{-1}).
- Carbonyl groups were determined using a commercial Kit (Protein Carbonyl Content Assay Kit – Ref MAK094. This methodology employs the use of 2,4-dinitrophenylhydrazine (DNPH) which leads to the formation of stable dinitrophenyl (DNP) hydrazine adducts, that can be detected spectrophotometrically. As described in the assay, the samples were previously treated with 10 µl of 10% streptozicin solution 100 µl of PMS and incubated during 15 minutes at room temperature, centrifuge at 13,000 g for 5 minutes. The supernatant was transferred and added to it a 100 µl of DNPH solution, vortex and incubated at room temperature during 10 minutes. Than were added 30 µl of 100% TCA solution, vortex and incubated for 5 minutes on ice. After centrifuge the samples at 13,000 g for 2 minutes and remove the supernatant, were added to the pellet 500 µl of ice-cold acetone. Then the samples were incubated during 5 minutes at-20°C, centrifuged at 13,000 g for 2 minutes and the supernatant was discarded. This step was repeated one more time. 200 µl of guanidine were added to the final pellet and sonicated briefly. Then the samples were incubated during 30 minutes

at 60°C and centrifuged during 2 minutes at 13,000 g. Finally, it is transferred 100 µl of each sample to the 96 well plate.

2.5. Protein quantification in the brain

Total protein contents both in PMS fraction and homogenate were determined according to the Biuret method (Gornall et al. 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard. Absorbance was measured at 550 nm using a SpectraMax 190 microplate reader.

Total protein content for carbonyl groups assay was determined in PMS, adjusted to a volume of 25 µl, using the BCA (Bicinchoninic Acid Kit) Assay Kit (Ref – BCA1 AND B9643) according to the principal of BCA assay using bovine serum albumin as a standard. Absorbance was measured at 562 nm using SpectraMax 190 microplate reader.

2.6. Data analysis

Statistical software (Statistica 8.0) was used for statistical analyses. All data were firstly tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) plus transformed by ln (x) whenever normality or homogeneity were not met. On normalized data a t-test was performed, otherwise a Man-Whitney U test was used to compare exposed and control samples in each time for mercury accumulation levels and for oxidative stress endpoints. Quotient between the values/activities of antioxidants in brain of exposed fish and control, as well as between levels of TBARS and carbonyl groups in exposed fish and control was also calculated.

3. RESULTS

No fish mortality was observed during both experiments. Though feeding was not strictly monitored, no alterations were perceptible during and after treatment on fish feeding behaviour.

3.1. Mercury levels in brain

Figure 6 presents the variation of total Hg (tHg) in brain of white seabream exposed to divalent Hg – Hg(II) (2 µg L^{-1}), as well as in control fish. tHg levels in brain differed significantly between control and exposed fish after 3, 7 and 14 days of exposure, as well as in both post-exposure periods (PE14 and PE28). Concentrations of tHg increased between E3, E7 and E14 ($t= 4.00$; $t= 4.93$; $t=5.82$ respectively, $p<0.05$), being the maximum values recorded in this last condition. tHg levels at PE14 and PE28 were identical to those found at E14 ($t= 1.17$; $t= 0.58$ respectively, $p<0.05$). Moreover, tHg levels in the post-exposure period never reached those found in control fish and, in general, no temporal variations were recorded for tHg levels in control fish.

Identically, just after 3 days of exposure to contaminated food by MeHg (8.7±0.5 µg g^{-1} dry weight), a significant higher accumulation of MeHg was found in the brain, in comparison with control (Figure 6). The accumulation of MeHg in brain increased gradually between 3 and 14 days of exposure ($t= 8.67$, $p<0.05$), when a maximum was reached. MeHg levels at E14 and PE14 were similar, while a significant decrease of MeHg levels was registered between E14 and PE28 ($t= 3.31$, $p<0.05$). As previously described for Hg(II) exposure, levels of MeHg in previously exposed fish never reached values of control in the post-exposure period, and MeHg in control brains were relatively constant along the experiment.

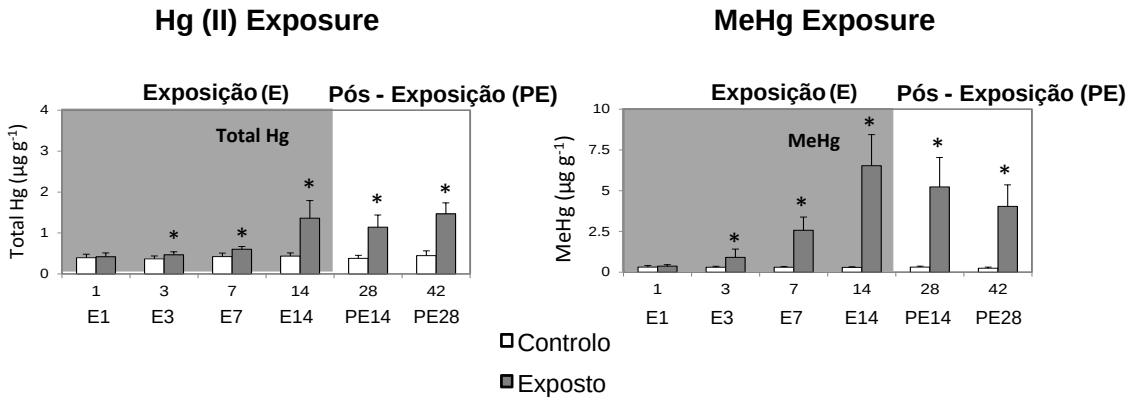


Figure 6 - Time variation of total Hg levels (tHg; µg g^{-1}) brain of white seabream along 14 days of exposure to Hg(II) (Experiment A) and MeHg (MeHg: ng g^{-1})(Experiment B) and 28 days of depuration (light area). Data correspond to mean ± standard deviation (n=8). Significant differences ($p<0.05$) in relation to the control group are indicated by * for each experiment time: 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days exposure, as well as 14 (PE14) and 28 (PE28) days post-exposure.

3.2. Oxidative stress in brain

Post-exposure to Hg(II), no changes on CAT activity were found along the experiment, while SOD increased significantly at E1 and E14 (Figure 7). On the other hand, exposure to MeHg was able to increase CAT activity at E1, E3, E14 and PE28. SOD activity also increased significantly at all experimental times, except E1 and E7.

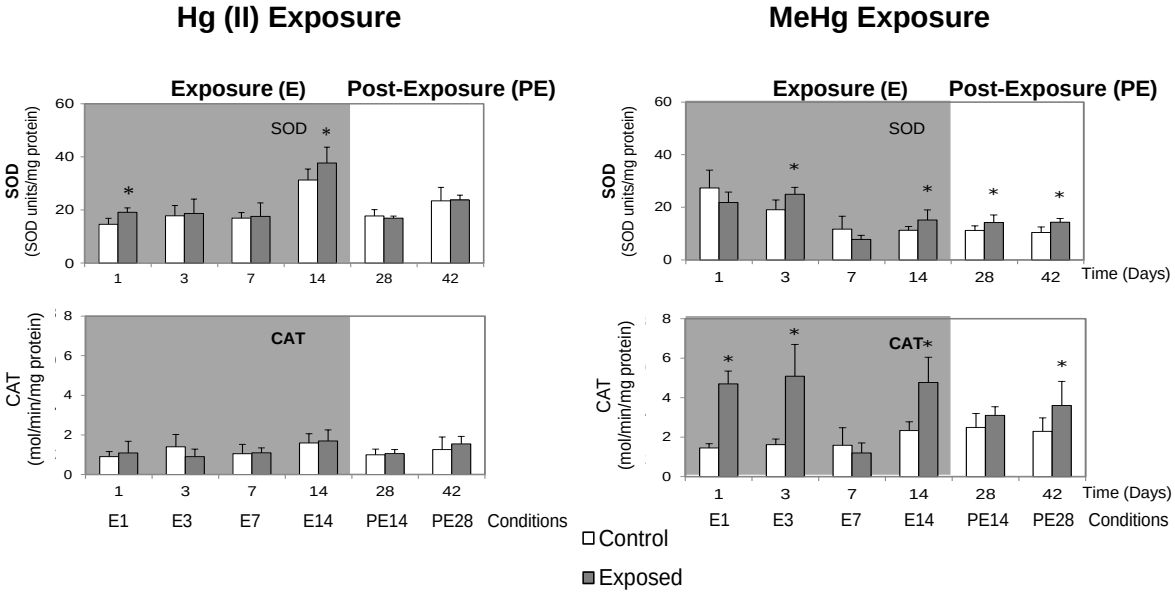


Figure 7 - Activities of superoxide dismutase (SOD) and catalase (CAT) in the brain of *Diplodus sargus* upon exposure to Hg(II) via water and MeHg via food. Mean and standard deviation are presented. Significant differences ($p<0.05$) in relation to the control group are indicated by * for each experiment time: 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days exposure, as well as 14 (PE14) and 28 (PE28) days post-exposure.

After exposure to Hg(II), GPx decreased significantly in the brain at E1, E7 and PE14, while at E14 an induction was found (Figure 8). Differently, MeHg only induced enhancements of GPx activities (E3 and E14). No significant changes of GST activities were found after Hg(II) exposure, whereas MeHg led to inductions along time (E1, E14, PE14 and PE28). Different temporal patterns were also found for GR in the two experimental conditions, namely no changes were found after Hg(II) exposure (except at E7 when an induction was found), while MeHg was on the basis of a GR induction at E3 and inhibitions at E7 and PE14.

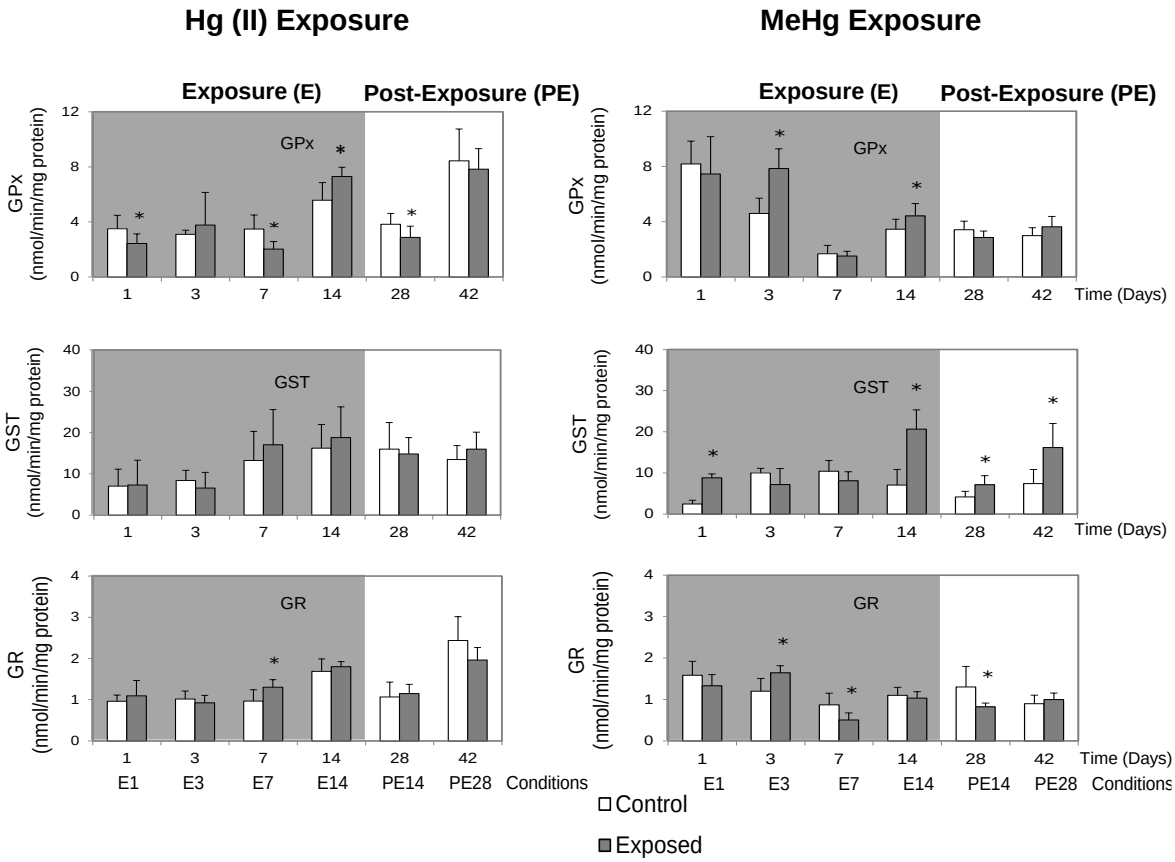


Figure 8 - Activities of glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) in the brain of *Diplodus sargus* upon exposure to Hg(II) via water and MeHg via food. Mean and standard deviation are presented. Significant differences ($p<0.05$) in relation to the control group are indicated by * for each experiment time: 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days exposure, as well as 14 (PE14) and 28 (PE28) days post-exposure.

Total glutathione content increased significantly in the last post-exposure period (PE28) upon exposure to Hg(II), meanwhile MeHg exposure led to a decrease of GSHt at E1 and to a significant increase at E14 (Figure 9).

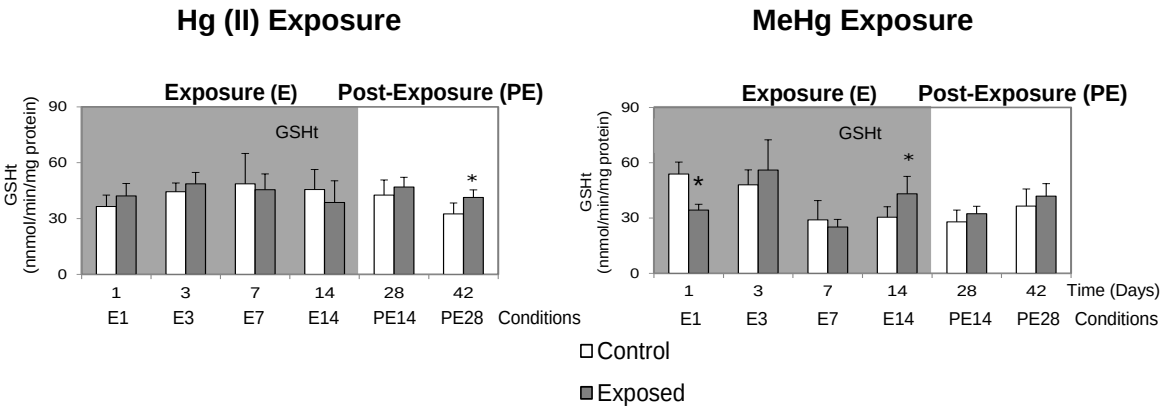


Figure 9 - Total glutathione (GSHt) levels in the brain of *Diplodus sargus* upon exposure to Hg(II) via water and MeHg via food. Mean and standard deviation are presented. Significant differences ($p<0.05$) in relation to the control group are indicated by * for each experiment time: 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days exposure, as well as 14 (PE14) and 28 (PE28) days post-exposure.

No lipid peroxidation occurred after exposure of fish to Hg(II), while an increase of carbonyl groups was recorded at E7 and PE14 (Figure 10). In general, MeHg did not elicit significant alterations on damage endpoints, except at E3 when an increase of LPO was found and at PE28 when a significant decrease of carbonyl groups occurred.

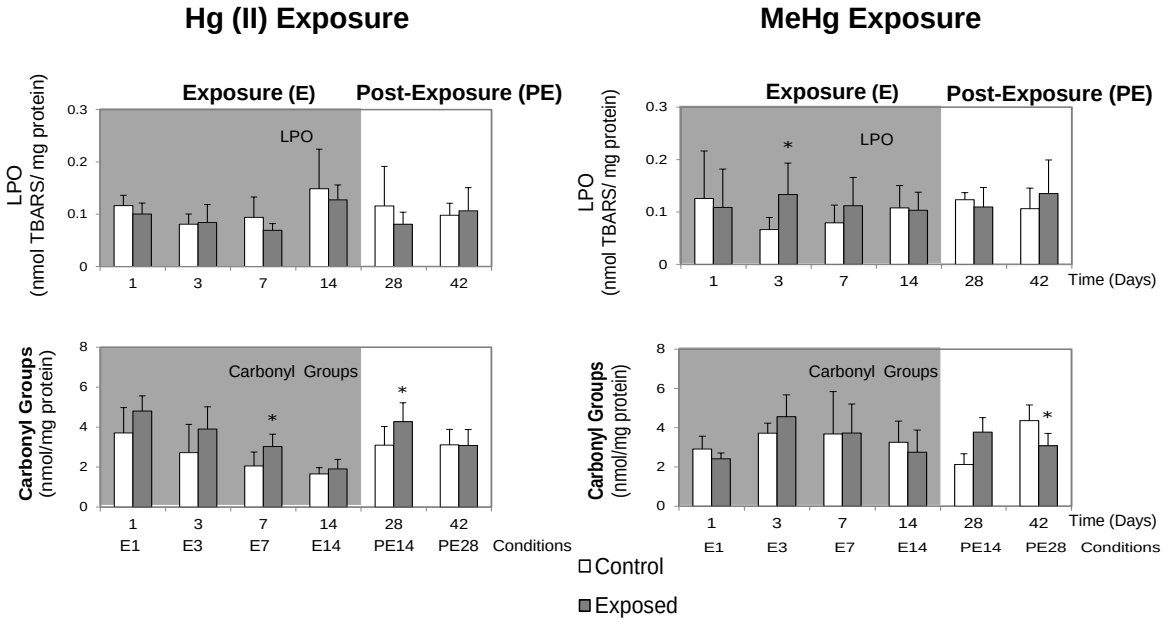


Figure 10 - Lipid peroxidation (LPO) and Carbonyl Groups levels in the brain of *Diplodus sargus* upon exposure to Hg(II) via water vs MeHg via food. Mean, standard deviation are presented. Significant differences ($p<0.05$) in relation to the control group are indicated by * for each experiment time: 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days exposure, as well as 14 (PE14) and 28 (PE28) days post-exposure.

3.3. Time-variation of antioxidant activities, GSHt, and damage indicators

The temporal variation of SOD, CAT, GST and GR activities, as well as GSHt levels (normalized to control values) followed different patterns for Hg(II) and MeHg (Figure 11). For instance, in the MeHg experiment, ratios of CAT and GST were higher than 1 at several experimental times, while for Hg(II) exposure ratios of those endpoints were always around 1. This is in line with the absence of significant differences between control and exposure conditions upon exposure to Hg(II) (as mentioned above). Besides that, it is very interesting to highlight that GPx ratios followed an identical temporal variation for data of experiment A (Hg(II) and experiment B (MeHg). Identical temporal patterns were also found for Hg(II) and MeHg exposures in the case of LPO and carbonyl groups ratios, even if ratios of carbonyl groups were mostly higher than 1 for Hg(II) and around 1 for MeHg.

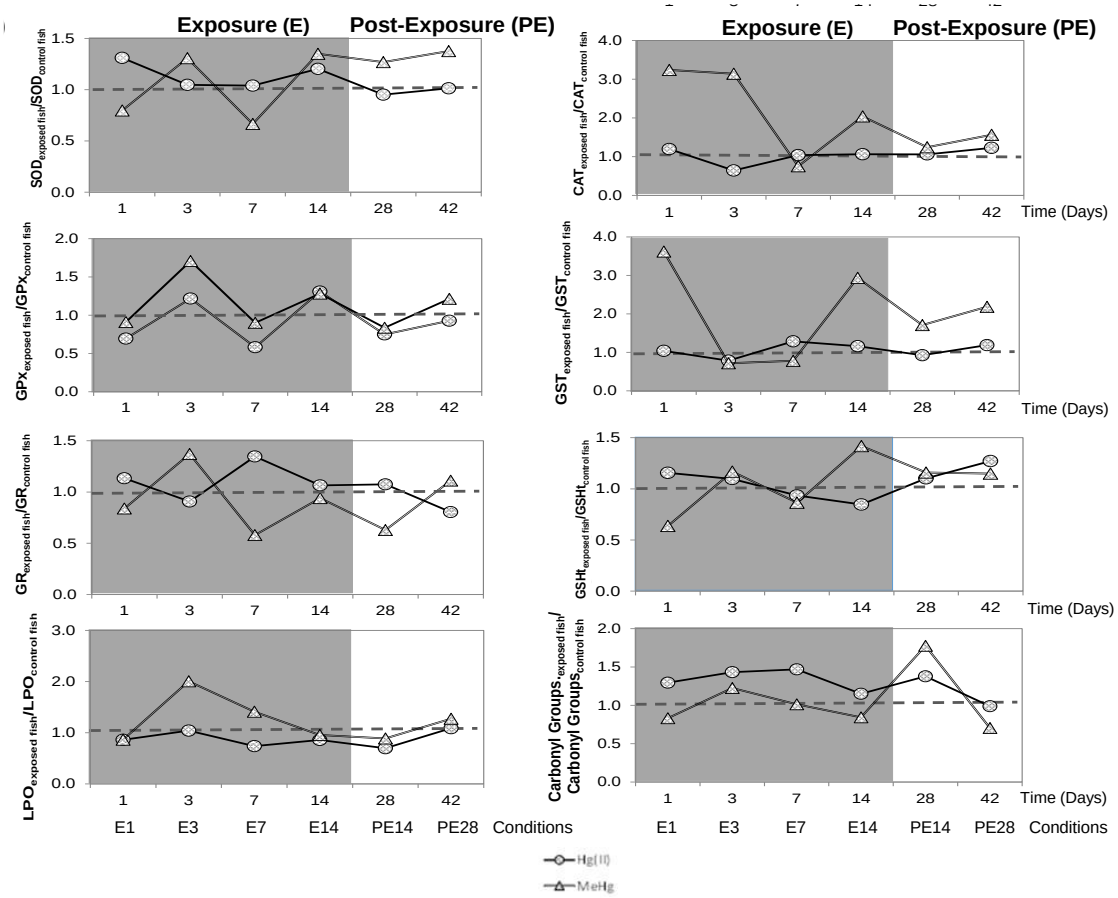


Figure 11 - Time variation of SOD, CAT, GPx, GST, GR activities and GSHt, LPO and Carbonyl groups levels calculated by the quotient between the mean of exposed and control fish within each sampling time. Results from both experiments are presented, namely: experiment A comprising Hg(II) exposure as circles; experiment B corresponding to MeHg exposure as triangles.

4. DISCUSSION

4.1. Hg(II) and MeHg accumulation in fish brain

The current study pinpointed that both Hg(II) and MeHg may reach fish brain after a short-time of exposure (only 3 days) to levels that can be found in water and food, respectively, at contaminated hotspots. In fact, these results are line with some field research performed in polluted areas, which also reported a high accumulation of Hg in fish brain (Mieiro et al. 2011; Pereira et al. 2014). Specifically, it was found that after a single meal of fish, MeHg absorption and deposition in human body is complete only within 3 days (Clarkson et al. 2007) this is in agreement with differences found for MeHg accumulation in *D. sargus*. In general, these studies missed the dynamic of Hg accumulation in fish brain over-time and its speciation, which are some of the scientific gaps that this study attempts to fill. In this context, it is very interesting to note that both Hg species increased significantly in the brain of *D. sargus* only after 3 days of exposure, suggesting a similar dynamic of uptake between these exposure conditions (experiment A - 2 µg of $HgCl_2$ L^{-1} in water; experiment B - 8.7±0.5 µg of MeHg g^{-1} in food, dry weight). The uptake of Hg(II) and MeHg was previously investigated in other fish species after waterborne and dietary exposure and in both cases the uptake rate constants estimated for MeHg were higher than those calculated for Hg(II) due to the lipophilicity and bioavailability (Wang & Wong 2003; Wang et al. 2010), which are divergent results from those presented here. The mechanism by which Hg(II) can reach the brain stills a controversial issue. Some works postulated that the BBB is impervious to Hg(II) and thus that it would reach the brain by axonal transport (Rouleau et al. 1999). Besides that, Hg(II) can also act as a direct BBB toxicant, affecting its structure and thus increasing its permeability to this toxicant (Zheng et al. 2003). Finally, a recent study suggested that Hg(II) can reach the brain after diffusion by BBB (Pereira et al. 2015), which is in line with current observations. Differently, the central nervous system represents the main target organ of MeHg toxicity reflecting its efficient transport into the brain. MeHg transport across the BBB, as well as its uptake by neural cells, occurs via a MeHg-L-cysteine complex that is transported by the L-type neutral amino acid transporter (Farina et al. 2013 and references herein).

Hg(II) and MeHg increased gradually in the brain along the exposure time, reaching a maximum after 14 days of exposure. Also, Feng et al. (2015) found an increase of both Hg species in zebrafish brain with time after dietary exposures, reaching maximum levels

after 62 days of exposure. Despite this identical temporal pattern in brain of *D. sargus*, MeHg was accumulated at much higher levels than Hg(II) in both exposure and post-exposure periods (Table 1). Fish were exposed to different levels via water (2 μg of $HgCl_2$ L^{-1}) and food (8.7±0.5 μg^{-1}), which prevents a rigorous comparison of both Hg forms in terms of its accumulation in brain. However, it can be stated that the exposure to MeHg via food promotes a higher accumulation of Hg in fish brain, pointing out the hazard of MeHg associated with trophic transfer. This hypothesis is in line with several studies that claimed that MeHg is very easily transferred through the food webs due to its lipophilicity (Morel et al. 1998; Wang & Wong 2003).

Table 1 – Accumulated levels of Hg(II) and MeHg (μg g^{-1}) in *Diplodus sargus* brain. The ratio between MeHg and Hg(II) accumulated in the brain at each experimental condition is also presented.

		Hg(II)	MeHg	Ratio
Exposure period	**E1**	0.417±0.094	0.436±0.107	≈
	E3	0.464±0.074	1.041±0.528	2x
	E7	0.603±0.064	3.216±0.599	5x
	E14	1.361±0.429	6.967±1.518	5x
Post-exposure period	**PE14**	1.143±0.295	6.793±2.757	6x
	PE28	1.465±0.273	3.509±0.599	2x

A very interesting result was found in the post-exposure period of this study, since Hg(II) levels did not change significantly along 28 days after exposure, whereas MeHg decreased considerable at PE28 (3.509±0.599 μg g^{-1}) in comparison with PE14 (6.793±2.757 μg g^{-1}) and E14 (6.967±1.518 μg g^{-1}). This result revealed that Hg(II) is well stable over time in the brain and this is mainly due to the formation of a complex with selenium (mercuric selenide – HgSe) that is very difficult to be eliminated by brain (Korbas et al. 2010). Complexation of Hg(II) as HgSe is considered a detoxification mechanism of Hg since it is a non-toxic and inert form of Hg in cells. Nevertheless, complexation of Hg(II) as HgSe can lead to a deficiency of essential Se-dependent enzymes (Dang & Wang 2011; Friberg & Mottet 1989). Moreover, Hg(II) may be sequestered by MTs, a family of cysteine-rich proteins that bind to metals (Cu, Zn and Hg) with high affinity (Ceccatelli et al. 2010), which difficult its elimination from brain cells. A slow elimination of several Hg counterparts by brain (including Hg (II)) was previously reported in zebrafish (Korbas et al. 2013) being in agreement with results of *D. sargus*. Current data pointed also that brain is a target-organ for Hg(II), as previously suggested for tilapia that accumulated significant levels of Hg(II) in the head at the end of 30 days of depuration (Wang et al. 2010). Hg(II) can cross BBB bi-directionally, but its influx and efflux from

brain is probably unbalanced, leading inevitably to its accumulation in brain over time, as previously described for Fe (Chen et al. 2014).

The considerable decrease of MeHg in brain of *D. sargus* at PE28 can be due to either the demethylation of MeHg to Hg(II) in brain or due to MeHg elimination. Indeed, results from a number of studies on humans exposed for many years to MeHg have shown high concentrations of Hg(II) in the brain in relation to total Hg pointing out to demethylation of MeHg in the brain (Friberg et al. 1989; Bjorkman et al. 2007). Similar evidence is available from long-term studies on monkeys exposed to MeHg (Vahter et al. 1995). Overall, previous works indicate that a significant accumulation of Hg(II) takes place with time despite the fact that the demethylation rate is slow (revision in Friberg & Mottet 1989). Although it hasn't been yet reported in fish brain before, demethylation of MeHg was demonstrated in bald eagle brain (Kalisinska et al. 2014).

The levels of MeHg in brain of *D. sargus* could also be reduced at PE28 due to its elimination, besides MeHg demethylation. In fact, *in vitro* studies performed by Kerper et al. (1996) with bovine brain capillary endothelial cells revealed that the complexation of MeHg with GSH and subsequent transport of the complex by an ATP-independent mechanism might be involved in the transport of MeHg out of brain capillary endothelial cells. This transport mechanism is responsible for the high mobility of MeHg. GSH complexation is a major cellular mechanism for MeHg excretion from the cell, protecting against MeHg toxicity (National Research Council 2000).

The detoxification of MeHg in fish brain is still unclear, although Branco et al. (2012) described that when fish were coexposed to MeHg and Se, the accumulation was much lower in all fish organs including in the brain, showing that selenium participates in the detoxification of MeHg (Korbas et al. 2010). It has also been stated that the chelating therapy, based on molecules constituted by –SH groups, could increase the excretion of MeHg (Farina et al. 2013) demonstrating that several thiol-containing complexing agents have been successfully used to remove MeHg from the organism (Clarkson 2002)

4.2. Alterations of the redox-defense system in brain upon accumulation of Hg(II) and MeHg

The cytotoxicity of methylmercury has been widely attributed to the induction of oxidative stress by either the overproduction of ROS or by the reduction of the oxidative defense capacity (Ceccatelli et al. 2010). The binding of MeHg to GSH due to its high affinity for SH-groups decreases the ability of this antioxidant to protect the cells from the free-radical mediated damage. Thus, the enhancement of oxidative stress upon MeHg accumulation in brain is quite well described for rodents (Choi et al. 1996; Stringari et al. 2008; Joshi et al. 2014), while it is less reported in fish (Berntssen et al. 2003; Mieiro et al. 2011). Brain is commonly reported as particularly sensitive to oxidative/free radical injury due to its high rate of oxidative metabolism, relatively low levels of antioxidant defenses, and high levels of polyunsaturated fatty acids and transition metal ions (Halliwell & Chirico 1993). Indeed, in *D. sargus* brain it was found an induction of SOD and CAT activities along with the exposure to MeHg (particularly at E3 and E14) and also in the post-exposure period (PE28) (Table 2). Both enhancements signaled a pro-oxidant challenge in the brain of the white seabream in line with MeHg accumulation. Indeed, SOD catalyses the dismutation of the superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), while CAT facilitates the removal of H_2O_2 that is metabolized to water and O_2. Several studies reported the induction of both enzymes in brain after MeHg exposure, namely in rats (Nabi et al. 2011) and fish (Berntssen et al. 2003). For instance, Berntssen et al. (2003) found a significant increase of SOD activities in the brain of salmon that were feed with 5 μg g^{-1} of MeHg. This dose induced protective redox defenses (as expressed by SOD induction) in the brain of salmon preventing lipid peroxidation. Berntssen et al. (2003) results are in line with data obtained for *D. sargus* that were exposed to a similar contamination level of MeHg in food (mean of 8.7 μg g^{-1}).

Table 2 - Synopsis of results obtained for oxidative stress after exposure to Hg(II) and MeHg. Activities induction or enhancements are signalised by ↑ while decreases or depletions are signalised by ↓.

		E1	E3	E7	E14	PE14	PE28
Hg(II)	SOD	↑			↑		
	CAT						
	GPx	↓		↓	↑	↓	
	GR			↑			
	GST						
	GSHt						↑
	LPO						
	Carbonyl Groups			↑		↑	
MeHg	SOD		↑		↑	↑	↑
	CAT	↑	↑		↑		↑
	GPx		↑		↑		
	GR		↑	↓		↓	
	GST	↑			↑	↑	↑
	GSHt	↓			↑		
	LPO		↑				
	Carbonyl Groups						↓

The induction of GPx at E3 and E14, coinciding with a significantly higher accumulation of MeHg in brain of *D. sargus*, is in agreement with CAT induction since GPx can also catalyze the reduction of hydrogen peroxide (among other peroxides). The induction of GPx also signaled a pro-oxidant challenge in the brain of *D. sargus* during exposure to MeHg. In fish, the principal peroxidase is a selenium-dependent tetrameric cytosolic enzyme (GPx) that employs GSH as a cofactor. GPx catalyses the metabolism

of hydrogen peroxide to water with the concomitant conversion of reduced glutathione (GSH) to its oxidized form - glutathione disulfide (GSSG). It has never been observed before the induction of GPx activity in fish brain upon MeHg exposure, although inhibitions are more frequently described in fish brain (Song et al. 2006; Mieiro et al. 2011).

Also the induction of GR at E3 pointed out a pro-oxidant challenge in the brain after MeHg accumulation. This is in line with several studies that reported elevated GR activities in organisms exposed to pro-oxidant stressors (Regoli et al. 2002; Branco et al. 2011). GR catalyzes the NADPH-depend reduction of the oxidized glutathione (GSSG) to GSH (Carlberg & Mannervik 1975). This enzyme is essential to maintain the adequate levels of cellular GSH, by maintaining a high GSH/GSSG ratio. In fish brain it was found an induction of GR after exposure to MeHg (Zemolin et al. 2012).

GST activities were also increased during some of the exposure times (E1 and 14), as well as in both post-exposure periods (PE14 and PE28). GSTs (a multigenic superfamily of multifunctional enzymes) may play a dual protective role associated with their activity on conjugation of electrophilic compounds (or phase I metabolites) with GSH (Van der Oost et al. 2003) and to a direct antioxidant action carried out by GST α-class catalyzing the reduction of organic hydroperoxides by GSH (Wang & Ballatori 1998). Given that the adopted methodology (using CDNB, which is conjugated by all GST isoforms with the exception of the q-class) determines total GST activity, the observed GST activity increases at the previous exposure conditions are difficult to interpret. Until now GST increased activity was never observed in fish brain upon exposure to MeHg.

Interestingly, total GSH only changed punctually in the brain during the exposure to MeHg and in different directions (i.e. at E1 it decreased significantly while at E14 increased). GSH can work as a carrier of mercury and an antioxidant. GSH can bind with MeHg reducing intracellular damage by preventing Hg from entering tissue cells and becoming an intracellular toxin (Kromidas et al. 1990). GSH-Hg complexes have been found in the liver, kidney, and brain, and appear to be the primary form by which mercury is transported and eliminated (Zalups 2000). The depletion of GSHt at E1 should be disregarded since at this exposure time no significant accumulation of MeHg was recorded in brain of exposed fish. On the contrary, MeHg accumulation peaked at E14 coinciding with the enhancement of GSHt pointing out an activation of antioxidant defenses due to MeHg. The protective effects of GSH are related to its activity as a buffer system that limits the amount of MeHg available for the interaction with sensitive macromolecules, and its ability as a ROS scavenger. Thus, the supply of GSH precursors to neurons via astrocytes and the maintenance of intracellular GSH concentrations are

critical to protect cells against MeHg-induced neurotoxicity. In addition to the antioxidant role of GSH, its conjugation with MeHg has been shown to be critical for MeHg efflux in different neural cell types. Thus, cells with higher GSH levels may have an enhanced elimination of intracellular MeHg with consequent higher resistance to its toxicity The enhancement of GSH levels upon exposure to MeHg and N-acetylcysteine simultaneously was previously observed by Kaur et al. (2006) in cell cultures of neurons and astrocytes. Being GSH a source of thiol groups and scavenger of free radicals (Aruoma et al. 1988) and the N-acetylcysteine an analogue of cysteine, which easily crosses the cell membrane and is rapidly deacetylated inside the cell to become available for GSH synthesis (Zafarullah et al. 2003),

The mechanism of Hg(II) toxicity in brain is not completely understood but some studies reported changes of oxidative stress related-endpoints. For instance, El-Demerdash (2001) reported lipid peroxidation in rat brain after exposure to $HgCl_2$. Moreover, *in vivo* exposure to $HgCl_2$ produced a significant decrease of the activities of several antioxidant enzymes in the cerebellum of rats (Hussain et al. 1997). The significant accumulation of Hg(II) in *D. sargus* brain did not lead to important alterations on antioxidants. Only punctually, some changes occurred, namely the induction of SOD at E1 and E14, as well as the induction of GR at E7 (Table 2). Also, in salmon brain no significantly changes were observed in SOD activity after exposure to a contaminated diet by 100 μg g^{-1} of Hg(II) (Berntssen et al. 2003).

GPx was the endpoint that fluctuated mostly upon exposure to Hg(II), with significant inhibitions at E1, E7 and PE14. GPx is an active scavenger of free radicals, and hence is strongly involved in protecting against potential cell injury and neuropathological conditions (Hussain et al. 1999). The significant decrease of GPx activities in white seabream brain at E7 and PE14 concomitantly with a high accumulation of Hg(II) disclose a breakdown of the antioxidant defense system. Depletion of GPx was previously found in brain of Atlantic salmon after dietary MeHg exposures (Berntssen et al. 2003). Activity of GPx also declined in a dose-dependent manner in the cerebellum of rat exposed to $HgCl_2$ (Hussain et al. 1999). As previously stated, Hg(II) is accumulated in the brain as a mercuric selenide that is per si an inert complex in the cells (Korbas et al. 2010). Nevertheless, the formation of this complex can interfere with enzymes that are dependent on Se such as GPx (Branco et al. 2012). In this context, it was also observed that a combined exposure of $HgCl_2$ and Se was followed by alleviated toxic effects of $HgCl_2$ on different antioxidant enzymes (El-Demerdash 2001). Hence, it should be

highlighted that Se could be able to antagonize the toxic effects of mercury (El-Demerdash 2001). Changes of GPx on *D. sargus* brain were also time-dependent since a significant induction was found at the end of the exposure period (E14), while inhibitions occurred at E1, E7 and PE14. Apparently, variations of GPx are not strictly related with the dose since at E14 and PE14 the same levels of accumulated Hg(II) were recorded. Effects of this Hg form in GPx seem to follow a non-monotonic pattern. This pattern is described as an inhibition in the enzymatic response at low doses and induction at higher toxic doses overcompensating the disturbance of homeostasis (Calabrese 2008). More specifically, low doses of Hg(II) induce inhibition followed by induction of the biomarker in higher doses, displayed in table 2. GPx activity represents a inverted U-shaped dose response, representing an affected biological system that responds to the damage in a compensatory manner during a lower concentration exposure, repairing damage in the process of re-establishing homeostasis (Calabrese 2008).

4.3. Oxidative damage upon Hg(II) and MeHg accumulation in the brain and its association with antioxidant defenses

Lipid peroxidation in fish has been widely used as a biomarker of oxidative stress (Guilherme et al. 2008a; Mieiro et al. 2010; Pereira et al. 2015), while measurement of protein carbonylation only recently has been applied to fish species for environmental health assessment (Almroth et al. 2005; Ferreira et al. 2005). In fact, the oxidation of polyunsaturated fatty acids is an important expression of the oxidative stress and has been pointed as a highly predictive biomarker of effect (Van der Oost et al., 2003; Guilherme et al., 2008a). Specifically, brain is highly vulnerable to lipid peroxidation (LPO) because it is a fatty rich organ. Thus, LPO has been proposed as an additional mechanism of Hg induced neurotoxicity (Yee & Choi 1996). Indeed, several studies with rats reported an increase of LPO after exposure to MeHg (Nabi et al. 2011; Joshi et al. 2014) and Hg(II) (Huang et al. 1996; Sener et al. 2003). Some studies also described increases of LPO in fish brain upon to MeHg (Berntssen et al. 2003). In general, in *D. sargus* brain no LPO occurred either after accumulation of Hg(II) and MeHg (except at E3 for the experiment with MeHg). It is very plausible that the strong activation of antioxidant defenses (SOD, CAT, GPx, GST) after the exposure to MeHg prevented lipid peroxidation. Depletion of GPx after Hg(II) exposure could lead to lipid peroxidation but this did not occur probably because other antioxidants (both enzymatic and non-enzymatic not measured) conferred resistance to lipid peroxidation. In fact, cysteine, alpha tocopherol and ascorbic acid, have a protective role against metals, as

demonstrated in the brain of rats (Patra et al. 2001). As previously claimed, GPx could be selectively inhibited associated with its Se-dependence, while other antioxidants kept their functionality (SOD, CAT, GR, GSH) provided protection to the brain cells.

Results of carbonyl groups after MeHg are consistent with those of LPO pointed out that the enhancement of antioxidant defenses prevented oxidative damage. On the contrary, in the experiment that comprised exposure to Hg(II) via water, a significant enhancement of carbonyl groups occurred at E7 and PE14 suggesting a break-down of antioxidant defenses. Actually, the accumulation of Hg(II) in the brain of white seabream was significantly higher in the brain of exposed fish at E7 and E14 than those of control. Carbonyl groups can be introduced in proteins by a number of different pathways, predominantly by metals catalysed oxidation (Almroth et al. 2005), and also by secondary mechanisms resulting from reactions of free radicals with other cellular constituents, such as lipids (Grune et al. 2003) where the highly oxidisable lipids may attack nearby proteins, causing the formation of an excess of protein carbonyl groups (Almroth et al. 2005). In this study, an increase of carbonyl groups was not followed by lipid peroxidation after exposure to Hg(II), pointing out to the first hypothesis. An increase in the number of carbonyl groups was previously well correlated with protein damage caused by oxidative stress (Shacter et al. 1994). The formation of carbonyl derivatives is non-reversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting in breakdown of proteins by proteases due to increased susceptibility (Almroth et al. 2005).

4.4. Contributions to the neurotoxicity of Hg(II) and MeHg in fish

The neurotoxicology of organic and inorganic Hg forms remains a matter of debate. While some authors claimed that the different forms of Hg share the same toxic entity, being toxicity dependent mainly on a differential bioavailability (De Flora et al. 1994), others stated that each Hg form has different physicochemical properties and toxicity profiles (Clarkson 1997). Currently there is a wider knowledge about MeHg neurotoxicity when comparing with Hg(II), this is due to the fact that MeHg is more liposoluble (Wang & Wong 2003; Wang et al. 2010) and because of that its higher affinity for crossing membranes and accumulate in the brain. Nevertheless, it has been proved that both forms can induce a wide range of neurotoxicological effects in brain of rat (Monnet-Tschudi et al. 1996; Aschner & Aschner 2007) and fish (Berntssen et al. 2003; Mieiro et al. 2010 and 2011). Moreover, behavioral changes in rat (Burbacher et al. 1990;

Fredriksson et al. 1992) and fish (Berntssen et al. 2003) were already reported after exposure to organic and inorganic mercury forms making worthy the clarification of neurotoxicity of MeHg and Hg(II) in fish.

Current results pointed out that both MeHg and Hg(II) can be accumulated in the brain of fish after exposure to realistic levels in contaminated areas of those compounds in food and water, respectively. Levels of MeHg in the brain were 2 to 6 fold higher than those of Hg(II), which is in line with its higher propensity to be accumulated in the brain (Mieiro et al. 2011; Pereira et al. 2014). Despite the higher accumulation of MeHg in brain in comparison with Hg(II), the activation of antioxidant defenses (SOD, CAT, GPx, GSHt, GST) prevented efficiently the occurrence of lipid peroxidation and the formation of carbonyl groups that is an indication of protein damage. On the contrary, the accumulation of Hg(II) in the brain of *D. sargus* was followed by an inhibition of GPx in two experimental times (E1 and E7). Such inhibition is probably the result of the formation of HgSe in the brain after the exposure to Hg(II) since GPx is a seleno-dependent enzyme (Branco et al. 2012). The depletion of GPx has been previously described as an indication of Hg-induced neurotoxicity in cerebellar granule cells (Farina et al. 2009) and *Salmo salar* brain (Berntssen et al. 2003) due to its role as a ROS scavenger (Battin & Brumaghim 2009). This inhibition of GPx was in turn probably on the basis the increase of carbonyl groups in the brain of *D. sargus* exposed to Hg(II).

While the majority of Hg-induced neurotoxicity are focused in MeHg, our study suggests that also Hg(II) need to be considered as a toxic element of the brain in fish. According to our results, this form can lead to oxidative damage while antioxidant protection prevented damage after MeHg exposure. Despite the lower accumulation levels of Hg(II) in the brain in comparison with MeHg, it led to the occurrence of oxidative damage on proteins. Overall, current results pinpointed the importance of considering Hg(II) as a toxicant of the fish brain.

5. CONCLUSIONS OF THE THESIS AND FURTHER PERSPECTIVES

The following conclusions were provided by this work:

1. MeHg was highly accumulated in the brain of *D. sargus* comparing to Hg(II) (2- to 6-fold higher), suggesting that MeHg is more efficiently transported to the brain than Hg(II). Despite that, MeHg and Hg(II) followed an identical time-variation pattern in the brain with both forms increasing significantly after 3 days of exposure.
2. Hg(II) was not significantly eliminated from the brain during 28 days of depuration, eventually due to the presence of the blood-brain barrier that does not facilitate Hg(II) efflux from the brain. On the contrary, upon 28 days of depuration, MeHg in the brain was significantly lower than at the end of the exposure period.
3. The accumulation of MeHg in the brain activated antioxidant defenses, while Hg(II) led to a significant inhibition of GPx, probably related with the formation of HgSe complexes in the cells.
4. Damage of lipids and proteins was efficiently prevented by the antioxidant defence system upon exposure to MeHg, while in Hg(II) the depletion of GPx was probably on the basis of oxidative damage.
5. Moreover, brain proteins seemed to be more susceptible to Hg(II) toxicity than lipids.
6. Finally, the slow elimination of Hg(II) by the brain can represent a risk for wild populations of fish and needs to be considered (together with MeHg) in the design of environmental health assessment plans.

This thesis contributed to a better knowledge of mercury neurotoxicology in fish, particularly by disclosing the potential neurotoxicity of Hg(II). However, to better compare the toxicokinetics of MeHg and Hg(II) in fish brain, organisms need to be exposed to both Hg counterparts together in an experiment with Hg isotopically marked. In experiment B, MeHg could be eventually demethylated in the liver (or even in the brain) and with the current design this was not demonstrable. The assessment of MeHg demethylation in fish can also be addressed by the employment of Hg isotopes. Besides that, it will be very interesting to clarify in what chemical forms Hg(II) is accumulated in the brain, namely the formation of HgSe and HgS. Moreover, further studies of Hg neurotoxicity in fish should complement biochemical effects in brain with fish behavioral alterations.

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